

Identification of the *Clostridium perfringens* Genes Involved in the Adaptive Response to Oxidative Stress

V. Briolat and G. Reyssset*

Unité de Génétique Moléculaire Bactérienne, Institut Pasteur, 75724 Paris Cedex 15, France

Received 5 September 2001/Accepted 29 January 2002

Clostridium perfringens is a ubiquitous gram-positive pathogen that is present in the air, soil, animals, and humans. Although *C. perfringens* is strictly anaerobic, vegetative and stationary cells can survive in a growth-arrested stage in the presence of oxygen and/or low concentrations of superoxide and hydroxyl radicals. Indeed, it possesses an adaptive response to oxidative stress, which can be activated in both aerobic and anaerobic conditions. To identify the genes involved in this oxidative stress response, *C. perfringens* strain 13 mutants were generated by Tn916 insertional mutagenesis and screened for resistance or sensitivity to various oxidative stresses. Three of the 12 sensitive mutants examined harbored an independently inserted single copy of the transposon in the same operon as two genes orthologous to the *ydaD* and *ycdF* genes of *Bacillus subtilis*, which encode a putative NADPH dehydrogenase. Complementation experiments and knockout experiments demonstrated that these genes are both required for efficient resistance to oxidative stress in *C. perfringens* and are probably responsible for the production of NADPH, which is required for maintenance of the intracellular redox balance in growth-arrested cells. Other Tn916 disrupted genes were also shown to play important roles in the oxidative stress response. This is the first time that some of these genes (e.g., a gene encoding an ATP-dependent RNA helicase, the β -glucuronidase gene, and the gene encoding the atypical iron sulfur prismane protein) have been shown to be involved in the oxidative response.

Clostridium perfringens is a ubiquitous, anaerobic, gram-positive bacterium that causes a number of diseases in humans and animals, including clostridial myonecrosis (or gas gangrene) and necrotic enteritis (reviewed by Rood and Cole [28]). Until now, the virulence of this species was attributed exclusively to production of effective toxins. *C. perfringens* synthesizes a number of major and minor toxins involved in the pathogenicity process. However, *C. perfringens* is considered an aerotolerant anaerobe that is capable of surviving in soil or arterial blood. It was recently shown that *C. perfringens* can escape from the phagosomes of macrophages in both aerobic and anaerobic conditions (22). This suggests that *C. perfringens* possesses a complex oxidative stress response that provides protection against the adverse effects of the reactive oxygen species encountered both in vivo and in vitro. *C. perfringens* possesses a classical Mn superoxide dismutase (26); however it is not clear whether this Mn superoxide dismutase scavenges superoxide radicals (38). No catalases have been found in *C. perfringens*. In contrast, components of the alternative oxidative stress protection system recently described in the sulfate-reducing bacterium *Desulfovibrio vulgaris* (17) have been identified in one strain of *C. perfringens* (15). This system consists of two non-heme proteins, rubrerythrin (Rbr) and rubredoxine oxidoreductase (Rbo). Rbo has superoxide reductase activity that catalyzes the reduction of $O_2^{\cdot-}$ to H_2O_2 without dismutation. Rbr is the terminal component of NADH peroxidase that catalyzes the reduction of hydrogen peroxide to water. The advantage of the Rbo-Rbr system is that there is no intracel-

lular production of molecular O_2 , which probably acts as a signal for growth arrest in anaerobic conditions (24, 40). Transcriptional analysis of the Rbr and superoxide dismutase genes of *C. perfringens* during the log and stationary phases, in both anaerobic and partially aerobic conditions, did not reveal the precise roles of the two proteins in the defense against oxygen (8).

Nonetheless, the ability of *C. perfringens* to survive when it is faced with a wide variety of natural and accidental oxidative stresses suggests that many genes are involved in this adaptive response, as is the case with other aerobic or facultatively anaerobic bacteria (for reviews see references 9 and 35). In an attempt to identify some of these genes, we used Tn916 insertional mutagenesis to generate *C. perfringens* strain 13 mutants (6), which were then screened for increased resistance or sensitivity to various experimental oxidative stresses (39). In this paper we describe identification of some of the genes implicated in the *C. perfringens* oxidative stress response and discuss their potential physiological roles.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1.

Media and growth conditions. *Escherichia coli* strains were grown aerobically at 37°C in Luria-Bertani broth containing ampicillin (100 μ g per ml), chloramphenicol (25 μ g per ml), or erythromycin (300 μ g per ml) when necessary. *C. perfringens* strains were routinely grown and stored under anaerobic conditions in TGY medium (27) or MCP medium (39). The medium was supplemented with chloramphenicol (25 μ g per ml), erythromycin (25 μ g per ml), or tetracycline (10 μ g per ml) when required. MMP medium (0.7 g of KH_2PO_4 per liter, 0.7 g of K_2HPO_4 per liter, 10 ml of a trace mineral solution per liter, 10 ml of a trace vitamin solution per liter) was used as a defined medium for the oxidative stress assays (2). The pH was adjusted to 7.0 with NaOH, and the medium was autoclaved in anaerobic conditions by using AutoPour bottles and closure technology (Amsco, Medical Products Division, Erie, Pa.). Before use, 0.5% (wt/vol) glucose, 0.5% (wt/vol) Casamino Acids, and 0.01% tryptophan were added

* Corresponding author. Mailing address: Unité de Génétique Moléculaire Bactérienne, Institut Pasteur, 25 rue du Docteur Roux, 75724 Paris Cédex 15, France. Phone: 33 1 45 68 83 08. Fax: 33 1 40 61 35 83. E-mail: greysset@pasteur.fr.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or relevant characteristics	Source or reference
<i>C. perfringens</i> strains		
13	Wild type	19
13R	Rifampin-resistant derivative of strain 13	39
<i>E. coli</i> DH5 α	<i>supE44</i> Δ <i>lacU169</i> (ϕ 80 <i>lacZ</i> Δ <i>M15</i>) <i>hsdR17</i> <i>recA1</i> <i>endA1</i> <i>gyrA96</i> <i>thi-1</i> <i>relA1</i>	10
Plasmids		
pUC19	Cloning vector, Amp ^r	41
pKNT19	Gram-positive/gram-negative shuttle vector, Amp ^r Emr ^r	1
pJIR418	Gram-positive/gram-negative shuttle vector, Amp ^r Clm ^r Emr ^r	33
pKNT20	Suicide vector, Amp ^r Emr ^r	This study
pKNT21	Suicide vector, Amp ^r Clm ^r	This study
pAM120	Amp ^r Tet ^r , contains Tn916	7
pKNT1280	pKNT19 recombinant containing a 7.72-kb insert, from the <i>EcoRI</i> DNA library of strain 13R ^a	This study
pKNT1300	pKNT19 recombinant containing a 7.68-kb insert, from the <i>EcoRI</i> DNA library of strain 13R ^b	This study
pKNTS1160	pKNT19 recombinant containing a 5.09-kb insert, from the <i>Sau3AI</i> DNA library of strain 13R	This study
pKNTS1030	pKNT19 recombinant containing a 4.54-kb insert, from the <i>Sau3AI</i> DNA library of strain 13R	This study
pUC1168	<i>ClaI</i> - <i>XbaI</i> fragment of pKNT1300 was cloned into the MCS ^c of pUC19	This study
pUC1171	1,168-bp <i>catP</i> cassette from pJIR418 was cloned into the unique <i>HpaI</i> restriction site of pUC1168 (within the <i>ycdF</i> ortholog-encoding gene)	This study
pUC2071	<i>PstI</i> - <i>XbaI</i> fragment of pUC1170 was cloned into the MCS of pKNT20	This study
pUC2072	650-bp <i>DraI</i> fragment from the <i>prismane</i> gene was cloned into the <i>SmaI</i> site of pKNT20	This study
pUC2073	450-bp <i>HindIII</i> fragment from the <i>ydaD</i> ortholog gene was cloned into the <i>HindIII</i> site of pKNT20	This study
pUC2174	580-bp <i>HincII</i> - <i>PvuII</i> fragment from the β -glucuronidase gene was cloned into the <i>SmaI</i> site of pKNT21	This study

^a See Fig. 3.^b See Fig. 4.^c MCS, multiple cloning site.

aseptically. MCP derivative medium was obtained by adding 0.1% (wt/vol) yeast extract to MMP medium. Anaerobic cultures were incubated at 37°C in an anaerobic chamber (Jacomex, Dagneux, France) in a 90% N₂-5% CO₂-5% H₂ atmosphere. Growth was assessed by measuring the optical density at 440 or 520 nm. For cell counting, aliquots from liquid cultures were serially diluted in anaerobically prepared 90 mM potassium phosphate buffer (PB) (pH 7.0) and plated onto prereduced MCP medium (containing 0.5% cysteine-HCl). Colonies (CFU) were counted after 24 to 48 h of incubation at 37°C. The number of colonies obtained was not affected by prior incubation for more than 6 h in PB.

Transposon mutagenesis. We introduced the suicide vector pAM120::Tn916 (7) into *C. perfringens* strain 13R by electroporation. Twelve independent transformations were performed using a saturating concentration of plasmid DNA (≥ 6 μ g). More than 2×10^4 independent tetracycline-resistant transformants were obtained in each experiment. The transformations were performed as described by Scott and Rood (32), with slight modifications. Briefly, the cells were cultured overnight in liquid TGY medium and diluted 100-fold in the same medium to an optical density at 440 nm of ≤ 0.4 . The cells were then washed in PB and resuspended in 0.1 volume of the same buffer before they were treated with lysostaphin (final concentration, 10 μ g/ml) at 37°C for 1 h. The cells were then centrifuged, washed twice with PB and once with SEB electroporation buffer (272 mM sucrose, 7 mM sodium phosphate buffer [pH 7.4], 1 mM MgCl₂), and resuspended in 1/50th of the initial volume of SEB. The cells (100 μ l) were mixed with plasmid DNA (0.1 to ≥ 6 μ g; final volume, ≤ 20 μ l), and the mixture was transferred into a 0.2-cm electroporation cuvette covered with an airtight seal. The electroporation cuvette was kept on ice for 10 min and then subjected to a single 6.25-kV/cm pulse delivered with a Gene Pulser apparatus (Bio-Rad Laboratories, Richmond, Calif.). The electric constants were 25 μ F, 400 Ω , and 6.25 kV cm⁻¹, which gave a time constant of about 6 ms. The electroporated cells were then resuspended in anaerobic conditions in 900 μ l of TGY medium containing 25 mM MgCl₂ and incubated for 60 to 75 min at 37°C before they were plated onto selective medium (solid TGY medium containing 25 mM MgCl₂ supplemented with the appropriate antibiotic). Independent transformants were recovered and reisolated twice on solid TGY medium containing the appropriate antibiotic.

Screening of Tet^r transformants for sensitivity to oxidative stresses. The Tet^r clones were grown independently in MCP medium supplemented with 0.5 μ g of tetracycline per ml until the mid-log phase (5×10^7 CFU/ml) and then spread onto solid MCP medium containing tetracycline. After 48 h at 37°C replica plating was performed with MCP medium containing tetracycline supplemented with H₂O₂ (1.4 mM), t-butylhydroperoxide (t-BH) (500 μ M), or rifampin (25 μ g/ml) as a control, and the cultures were incubated at 37°C in anaerobic conditions. Alternatively, the clones were replicated on MCP medium containing tetracycline supplemented with plumbagin (PL) (2 or 2.5 μ M) and rifampin (25 μ g/ml) and exposed to air for 2 h prior to incubation at 37°C in anaerobic conditions. After 48 h, the clones that were unable to survive under one or more of the oxidative stress conditions (42 of 8,500 clones tested) were reisolated. The MICs of H₂O₂, t-BH, and PL were determined by incubating these 42 clones in solid MCP medium containing 1.00, 1.25, 1.50, 1.75, or 2.00 mM H₂O₂; 250, 500, 750, or 1,000 μ M t-BH; or 0.50, 0.75, 1.00, or 2.50 μ M PL.

Screening of Tet^r transformants for resistance to oxidative stresses. The Tet^r clones were grown independently in liquid MCP medium supplemented with 0.5 μ g of tetracycline per ml until either the mid-log phase (5×10^7 CFU/ml) or the stationary phase (5×10^8 CFU/ml). They were then spread onto solid MCP medium containing tetracycline supplemented with H₂O₂ (2 mM), t-BH (1 or 2 mM), or ethanol (11%) and incubated in anaerobic conditions at 37°C for 48 h. Cells were also spread onto solid MCP medium containing tetracycline supplemented with H₂O₂ (1.8 mM), t-BH (1 mM), ethanol (10%), or PL (10 or 50 μ M) and exposed to air at 37°C for 2 h before incubation in anaerobic conditions. The clones that were able to grow on the selective plates were reisolated, and the MICs were determined as described above. About 40 candidates were selected by this procedure.

Exposure to air in liquid medium. Bacteria were grown to the mid-exponential or early stationary phase in MMP medium at 37°C in an anaerobic chamber. The bacterial cultures were then transferred to aerobic conditions at 37°C with shaking in a rotary incubator (250 rpm) in flasks with a capacity that was 5 to 10 times greater than the volumes of the cultures. At various times, aliquots were returned to the anaerobic chamber, and viability was measured as previously described.

The cell survival rate was calculated by dividing the number of viable cells after exposure to air by the total number of viable cells before treatment.

Exposure to other types of oxidative stress in liquid medium. Bacteria were grown to either the mid-exponential or stationary phase in MMP medium. Aliquots were treated with various concentrations of H₂O₂ or t-BT and incubated at 37°C in anaerobic conditions for 2 h. The survival rate was calculated by dividing the number of viable cells after exposure to H₂O₂ or t-BT by the total number of untreated cells at the same time. The response to PL was analyzed only with a mid-exponential-phase culture. After various concentrations of PL were added in the anaerobic chamber, the cells were exposed to air with vigorous shaking for 1 h. Finally, they were transferred to the anaerobic chamber, and the number of viable cells was determined as described above.

Oxidative stress assays in solid medium. Cells were grown to the mid-log or early stationary phase in MMP or MCP medium, and 200 µl was spread onto MCP medium. When the test was performed in anaerobic conditions, 5-µl portions of 50 or 100 mM H₂O₂ or 5-µl portions of 12.5 or 25 mM t-BT were spotted onto small circles of blotting paper that had been placed on the agar plates. The plates were then incubated for 24 h at 37°C. The sensitivities to the oxidative compounds were also tested after exposure to air. In this case, 5-µl portions of 6.25 or 12.5 mM PL, 25 or 50 mM H₂O₂, or 12.5 or 25 mM t-BT were spotted onto the membranes. The plates were kept in anaerobic conditions for 30 min, exposed to air at 37°C for 0.5 h, and incubated for 24 h at 37°C in an anaerobic chamber. In each case, the inhibition zones surrounding the filters were measured to compare sensitivities. For each mutant, three or four diameters were measured for each product and each experimental condition. The results were compared with those obtained with the wild-type strain tested the same day in the same conditions.

β-D-Glucuronidase assay. β-Glucuronidase activity was measured as described by Dupuy and Sonenshein (5).

Construction of the *C. perfringens* chromosomal DNA libraries. Chromosomal DNA libraries of *C. perfringens* strain 13R were constructed by using the shuttle vector pKNT19 (1). *C. perfringens* DNA was prepared as follows. The strain was cultivated in 200 ml of MCP medium extract until the optical density at 440 nm was 0.7. The cells were centrifuged, and the pellet was resuspended in lysis buffer (25 mM Tris-HCl [pH 8.0], 25 mM saccharose, 10 mM EDTA, 1 mg of lysozyme per ml). After 15 min at 37°C, sodium dodecyl sulfate was added at a final concentration of 1%, and the mixture was incubated at 55°C until cell lysis was complete (about 20 min). RNase (final concentration, 100 µg/ml) was then added, and the mixture was incubated at 37°C for 1 h. Proteinase K (final concentration, 500 µg/ml) was then added, and the mixture was incubated at 55°C for 2 h. Two phenol-chloroform-isoamyl alcohol (25:24:1, vol/vol/vol) extractions and a chloroform-isoamyl alcohol (24:1, vol/vol) extraction were then performed. After ethanol precipitation, the DNA was resuspended in 1 ml of 10 mM Tris-HCl (pH 8.0). About 10 µg of total DNA was partially digested with *EcoRI* or *Sau3AI*, and the 6- to 20-kb fragments were recovered from a 10 to 40% sucrose density gradient. The restriction fragments (average size, 9 kb) were then ligated into the dephosphorylated *EcoRI* or *BamHI* restriction sites of pKNT19 and introduced into *E. coli* DH5α by electroporation (10). At least 3,000 clones were pooled to obtain the pKNT100 (*EcoRI*) and pKNTS100 (*Sau3AI*) plasmid banks.

Molecular and genetic techniques. Unless otherwise noted, all DNA manipulations were performed by standard techniques (29). Inverse PCR was used to isolate some of the Tn916-flanking regions of the mutants (18). Depending on the restriction sites present in the *EcoRI-EcoRI* fragments containing Tn916, the mutant chromosomal DNA was digested with *HindIII* and/or *BclI*. The primers used for amplification were primers Hd554 (5'-GGCATGTAGAATAAG-3') and Hd555 (5'-GGTTGGATCCGTCCTCGTTGTA-3') or primers Bclr (5'-GTCTAGACAACGTCCAGACGC-3') and Belga (5'-CCTGGATCCAGTGTGATAAGTC-3'), depending on which enzyme was used to digest the DNA. The prisma-encoding gene was amplified by using primers PrisCPG (5'-AGGTAACCTTAGTTACCGAG-3') and PrisCPD (5'-ACACAGCTGTGTAGTCTTC-3') and then cloned into pJIR418 (33).

Insertional mutagenesis. Two suicide vectors (pKNT20 and pKNT21) were constructed for gene inactivation. pKNT20 was constructed by cloning a blunt-ended *SacI-NsiI* restriction fragment from pKNT19 containing the *ermC* determinant of pIM13 (20) into the *SspI* restriction site of pUC19. pKNT21 was constructed by cloning a 1,173-bp *SmaI* restriction fragment containing the *catP* determinant of plasmid pJIR418 into the *SspI* restriction site of pUC19. The *ycdF* ortholog was inactivated by using plasmid pUC2071 as a suicide vector and selecting Clm^r-Emr^s transformants. The genes encoding prisma, alcohol dehydrogenase (*ycdD* ortholog), and β-glucuronidase were inactivated by simple recombination with selection for Emr^r or Clm^r transformants after electroporation of strain 13R with pUC2072, pUC2073, or pUC2174. Replacement of the

wild-type *ycdF* ortholog gene was confirmed by PCR. Disruption of the three other genes was confirmed by Southern hybridization using pUC19 as a probe (data not shown).

Nucleotide sequence determination. Both strands of the cloned fragments were sequenced with an ABI373 automated sequencer. The Genetics Computer Group program (University of Wisconsin, Madison) was used for computer analysis.

Nucleotide sequence accession numbers. The DNA sequences of ORF1 to ORF7 and ORFA to ORFC have been deposited in the EMBL, GenBank, and DDBJ nucleotide sequence databases under accession numbers AJ420783 and AJ420784, respectively.

RESULTS

Selection of Tn916 mutants with altered oxidative stress response. Tn916 mutagenesis was used to isolate *C. perfringens* mutants with altered oxidative stress response. The Tn916-containing suicide vector pAM120 was introduced into a rifampin-resistant derivative of *C. perfringens* strain 13 by electroporation (7). Twelve transformations were performed, which led to 12 banks containing about 2×10^4 independent Tet^r clones. These clones were then screened, and Tet^r mutants with higher or lower sensitivities (altered MICs) to air, a superoxide-generating compound (PL), or to hydrogen peroxide and to peroxy radical-generating compounds (H₂O₂, ethanol, and t-BT) were identified. About 80 potential candidates were selected, and their phenotypes were characterized by rapid plate test techniques.

Tn916 can randomly insert itself into the chromosomes of the most gram-positive bacteria, even though insertion hot spots exist (18). Multiple insertion events have also been described. The copy numbers of Tn916 in 42 of the 80 Tet^r clones examined and the physical maps of their insertion regions were therefore determined by Southern hybridization. Tn916 possesses unique *BclI* and *HindIII* restriction sites but no *EcoRI* sites. DNA was purified from each mutant and digested with each of the three enzymes independently or with *EcoRI* plus *BclI* or *EcoRI* plus *HindIII*. The resulting fragments were separated by gel electrophoresis and transferred onto a nylon membrane. The blot was then hybridized using a uniformly labeled Tn916 probe (data not shown). Only 12 of the 42 samples tested contained a single copy of Tn916.

Preliminary phenotypic characterization of the selected mutants. Eight of the 12 mutants were chosen for further study. They were separated into two classes. The first class, containing the resistant mutants, included mutants 13052, 13055, 13095, and 13133. They were selected for their ability to survive and then grow on solid medium containing high concentrations of superoxide and/or hydroxyl or organic peroxy radical-generating compounds. The second class, containing the sensitive mutants, included mutants 13050, 13096, 13521, and 13543, which did not grow, or grew less efficiently than the parent strain, on at least one of the selective solid media. To characterize the mutants further, we grew them to the mid-log phase in anaerobic conditions in MMP medium and then evaluated their sensitivities to air and to different concentrations of PL, H₂O₂, and t-BT (Fig. 1 and 2). Strain 13052 was the only resistant mutant that was resistant to peroxide stress but not to superoxide. The other resistant strains were more or less resistant to both peroxide and superoxide stresses. All of the PL-sensitive mutants except one (strain 13521) were also more sensitive to H₂O₂ stress than the wild-type strain.

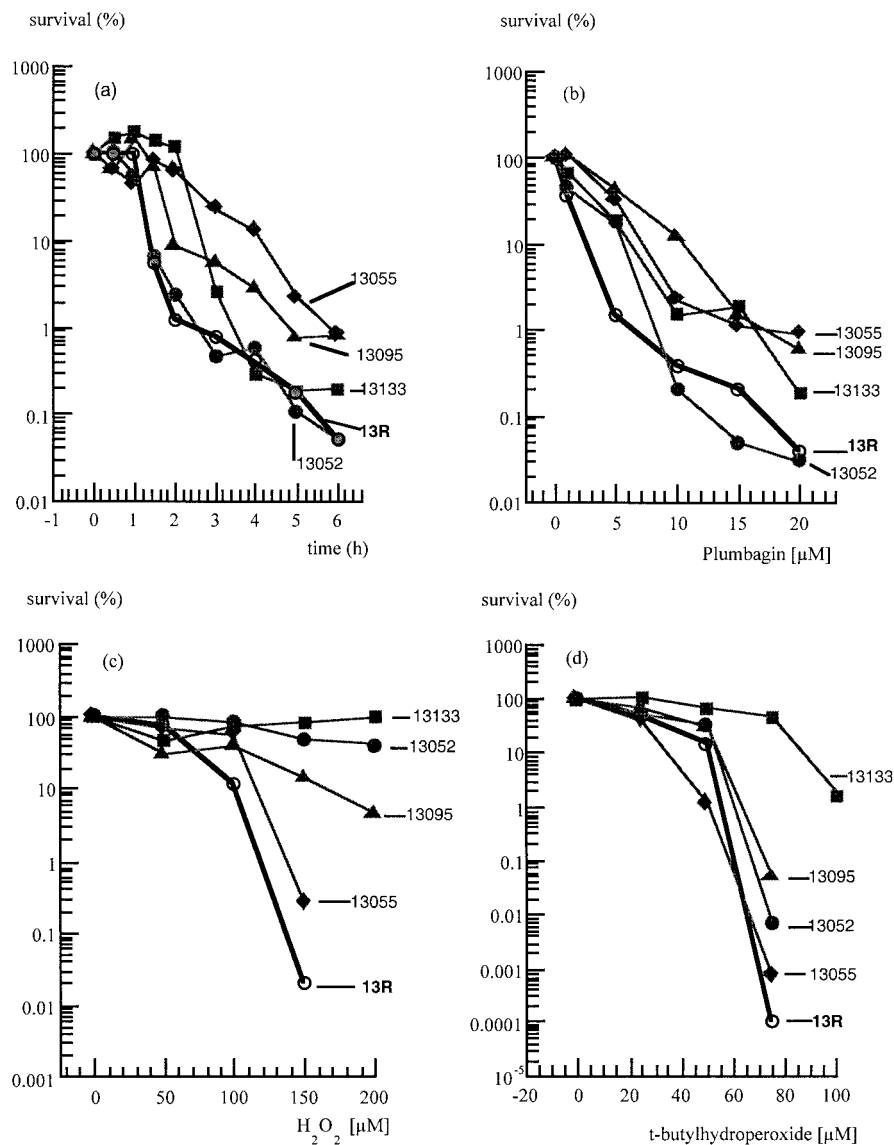


FIG. 1. Sensitivities of the resistant mutants and wild-type *C. perfringens* strain 13R to air (a), PL (b), H₂O₂ (c), and t-TB (d). The results are means based on two experiments. Symbols: ○, strain 13R; ●, mutant 13052; ◆, mutant 13055; ▲, mutant 13095; ■, mutant 13133.

Cloning of the Tn916 insertional region. Two strategies were used to clone the Tn916-flanking regions of the mutants. First, the *EcoRI-BclI* or *BclI-BclI* chromosomal restriction fragment adjacent to the Tn916 copy was cloned directly. We exploited the large size of the transposon (18 kb) to enrich for the population of high-molecular-weight *EcoRI-EcoRI* restriction fragments on a sucrose gradient prior to the pUC19 cloning experiments. The use of the colony hybridization technique with uniformly labeled Tn916 as the probe meant that analysis of about 400 recombinant clones was usually sufficient to detect the *EcoRI-BclI* or *BclI-BclI* restriction fragment including part of the transposon. The second technique was based on an inverse PCR approach. Two sets of Tn916-specific primers (primers Hd555 and Hd554 and primers Bcldr and Bclga) were used to amplify the regions adjacent to the left and right ends of Tn916. The nucleotide sequences of the flanking regions

were then determined, and for some mutants the equivalent region from the wild type was also sequenced using appropriate clones from the *Sau3AI* and/or *EcoRI* genomic banks of *C. perfringens* strain 13. The Tn916 insertion site was then precisely characterized for the selected mutants.

Location of Tn916 in the sensitive mutants (strains 13050, 13096, 13543, and 13521). The unique copy of Tn916 was inserted in the same chromosomal region in strains 13050, 13096, and 13543. This region was partially covered by pKNTS1160, which contained a 4.5-kb *Sau3AI* fragment, and pKNTS1280, which contained a 7.7-kb *EcoRI* restriction fragment (Fig. 3). The entire 7.7-kb region was sequenced and was found to contain five complete open reading frames (ORFs) and one incomplete ORF at each end of the region. The first incomplete ORF (ORF1; nucleotides [nt] 2 to 454) potentially encodes the C-terminal 130 amino acids of a polypeptide that

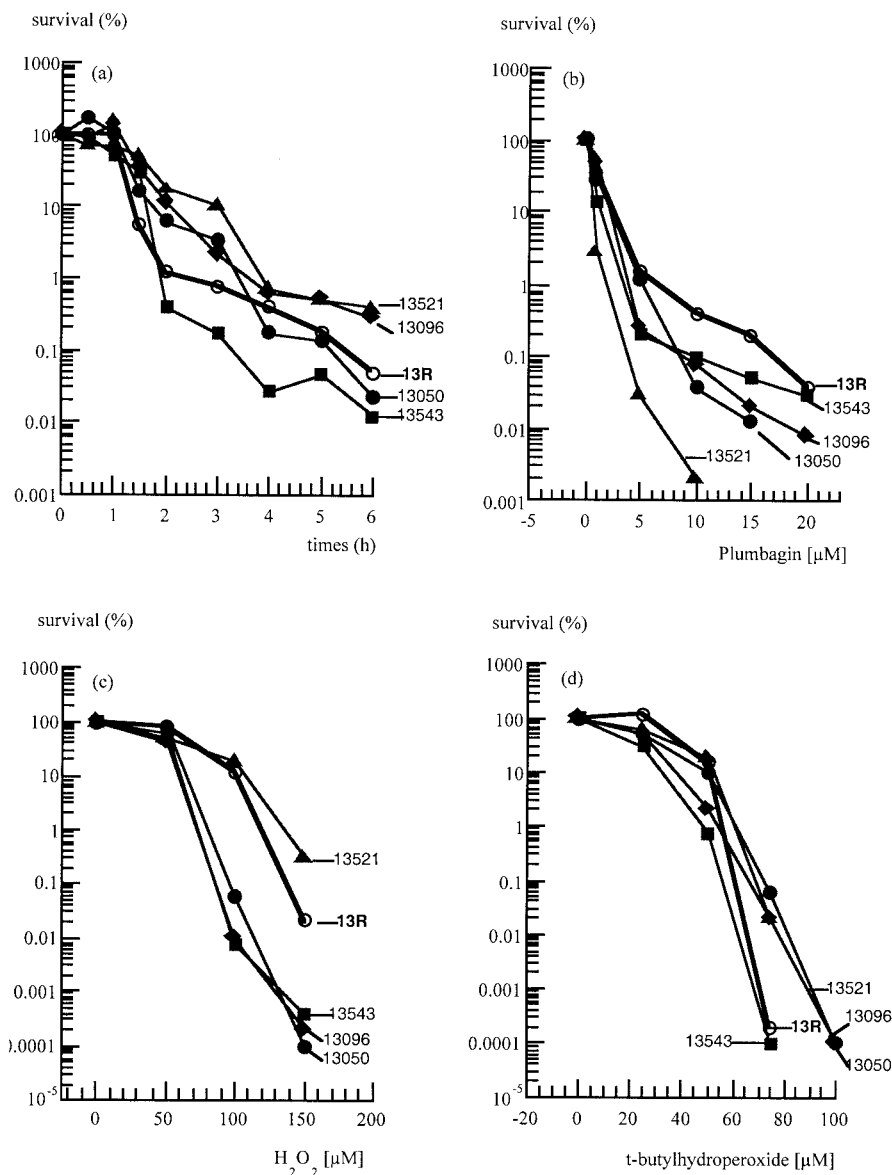


FIG. 2. Sensitivities of the sensitive mutants and wild-type strain 13R of *C. perfringens* to air (a), PL (b), H₂O₂ (c), and t-TB (d). The results are means based on two experiments. Symbols: ○, strain 13R; ●, mutant 13050; ◆, mutant 13096; ■, mutant 13543; ▲, mutant 13521.

exhibits 40% sequence identity with a conserved hypothetical protein from various bacteria, including *Vibrio cholerae* (11), *Bacillus halodurans* (36), and *Bacillus firmus* (accession number P30267). The second ORF (ORF2; nt 782 to 2425) encodes a 547-amino-acid protein that exhibits high levels of identity with the hybrid cluster [4Fe-2S-3O] prismane protein found in a large number of bacteria, including *Desulfovibrio desulfuricans* (65% identity; accession number S24389), *Desulfovibrio vulgaris* (65% identity; accession number S29861), *Methanococcus jannaschii* (58% identity; accession number E64395), and *E. coli* (41% identity; accession number A64826). Three stop codons were present downstream of ORF2, as was a 21-bp hairpin loop structure (nt 2464 to 2507) that might act as a transcription terminator. The following ORF (ORF3; nt 2809 to 4101) is transcribed in the orientation opposite that of

ORF2 transcription. ORF3 is located 389 nt downstream of the stop codon of ORF2 and encodes a conserved hypothetical protein of unknown function present in a various gram-positive bacteria. ORF4 (nt 4286 to 5059) and ORF5 (nt 5123 to 6049) are located close to each other and appear to belong to the same transcriptional unit. In strains 13543, 13050, and 13096, the transposon is inserted in ORF4 at nt 4501, 4502, and 4504, respectively. ORF4 encodes a hypothetical 257-amino-acid protein, which possesses a short-chain alcohol dehydrogenase oxidoreductase signature. The protein encoded by ORF5 probably also has dehydrogenase activity. The gene encoding ORF5 is present in a wide variety of bacteria, including *Pseudomonas aeruginosa* (53% identity; accession number G83378) and *Bacillus subtilis* (51% identity; accession number D69768). The protein encoded by ORF5 exhibits 51% identity with the glu-

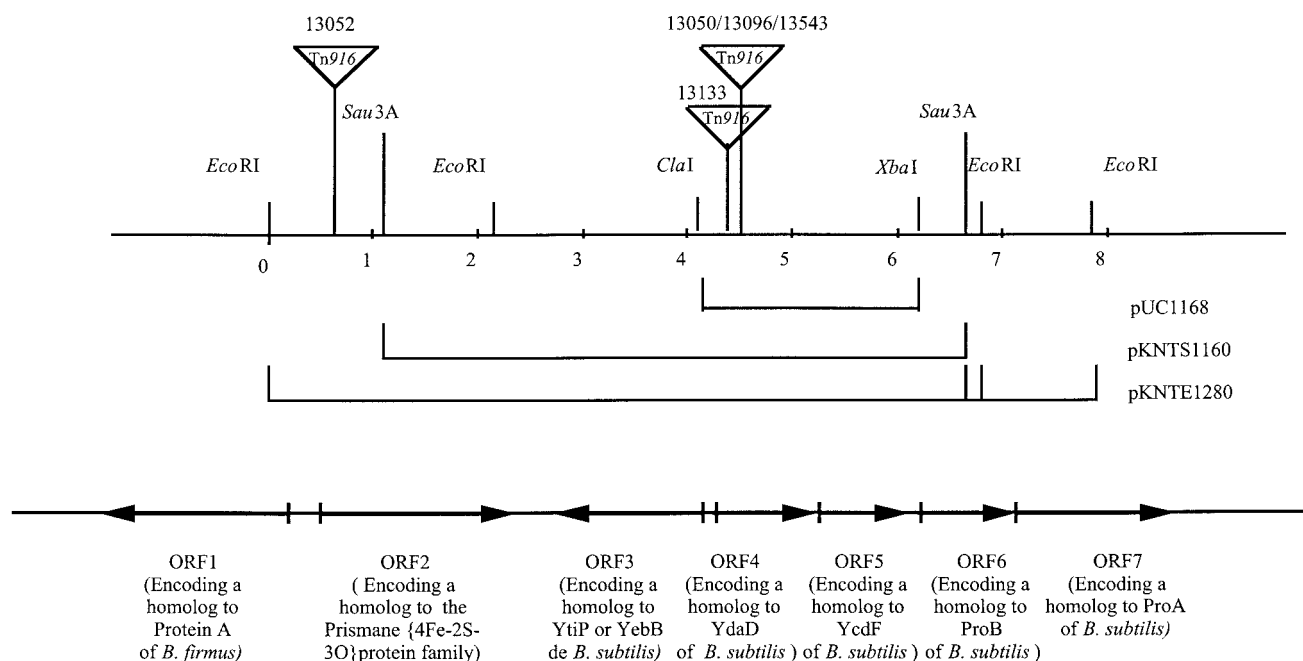


FIG. 3. Partial restriction map of pKNTE1280 and pKNTS1160 showing the location of Tn916 in mutants 13052, 13050, 13096, 13543, and 13133. The solid arrows indicate the locations of the various ORFs identified by sequence analysis. The scale is in kilobases.

cose-1-dehydrogenase (EC 1.1.1.47) of *Bacillus megaterium*. In *B. subtilis*, the orthologs of ORF4 and ORF5 (*ydaD* and *ycdF*, respectively) were recently shown to be induced by different stress conditions, including heat shock, oxidative stress, glucose limitation, and oxygen limitation, and to be members of the sigma B regulon (25). Finally, downstream of ORF4 and ORF5 are two genes (ORF6 and ORF7, equivalent to *proA* and *proB* of *B. subtilis*) which have been implicated in the synthesis of proline.

In 13521 the transposon was detected on a region of the chromosome covered by pKNTS1030, which contained a 5.0-kb *Sau3AI* fragment, and pKNTE1300, which contained a 7.5-kb *EcoRI* fragment (Fig. 4). The two overlapping restriction fragments were sequenced by subcloning in the pUC19 vector. The region was found to contain one incomplete ORF (ORFA), followed by four complete ORFs (Fig. 4). The transposon was inserted between ORFB and ORFC in 13521. The left end of Tn916 was located 45 nt upstream of ORFB, and the right end was located 15 nt downstream of the stop codon of ORFC. ORFB (nt 560 to 2359) encodes a polypeptide that exhibits 66% identity with a β -glucuronidase gene from *Staphylococcus* sp. (accession number AF354044), and its product exhibits 47% identity with the β -glucuronidase (accession number P05804) encoded by the *uidA* gene of *E. coli*. The product encoded by ORFC (nt 2423 to 3448) exhibits 33% identity over 266 amino acids with a 2-keto-3-deoxygluconate kinase from *Thermotoga maritima* (accession number G72422).

Location of Tn916 in the resistant mutants (strains 13052, 13055, 13095, and 13133). Tn916 was located in the middle (nt 651 of 1,533 nt) of a gene encoding a probable ATP-dependent RNA helicase, which possesses a typical DEAD box motif (D-E-A-D, or Asp-Glu-Ala-Asp), in strain 13055 (16). The deduced polypeptide exhibits 53% identity with the cold shock

RNA helicase protein (accession number P33906) of *Klebsiella pneumoniae* and 50 to 59% identity with the products of the *ydbR* and *deaD* genes of *B. subtilis*. In 13095, Tn916 was inserted 27 nt downstream of the *tfh* gene encoding a 10-formyltetrahydrofolate synthetase polypeptide in *C. perfringens* (accession number X86514) and 142 nt upstream of a gene exhibiting a high degree of identity (65% identity in a 114-amino-acid overlap) with the *yacB* gene from *B. subtilis*, whose function is still unknown.

In 13052 and 13133, the transposon was also found in the DNA region covered by pKNTS1160 and pKNTE1280 (Fig. 3). The Tn916 of mutant 13052 was inserted 234 nt downstream of the theoretical start codon of ORF1 and 34 nt upstream of the ATG initiation codon of the prismane gene (ORF2). In 13133, the copy of Tn916 was inserted in ORF4 (nt 4476) but was integrated in the orientation opposite that in 13050, 13096, and 13543.

Role of the *C. perfringens ydaD* and *ycdF* ortholog genes in the oxidative stress response. We performed complementation and knockout gene experiments to confirm that ORF4 (*ydaD* ortholog) and ORF5 (*ycdF* ortholog) play a role in the oxidative stress response in *C. perfringens*. For the complementation experiments, the 13R strain and the 13096 mutant, which harbors the Tn916 element within the *ydaD* ortholog, were transformed with either pKNTE1280, harboring wild-type *ydaD* and *ycdF*, or pKNT19 as a control. The sensitivities of the mutants to H₂O₂ and t-BT in anaerobic conditions were tested. Introduction of pKNTE1280 into strain 13096 rendered it as resistant to H₂O₂ and t-BT as the wild-type strain harboring the control plasmid, 13R/pKNT19 (Fig. 5). Moreover, the presence of pKNTE1280 in strain 13R significantly enhanced its resistance to H₂O₂ compared to the resistance of the control (13R/pKNT19). It was also found that strain 13R/pKNTE1280

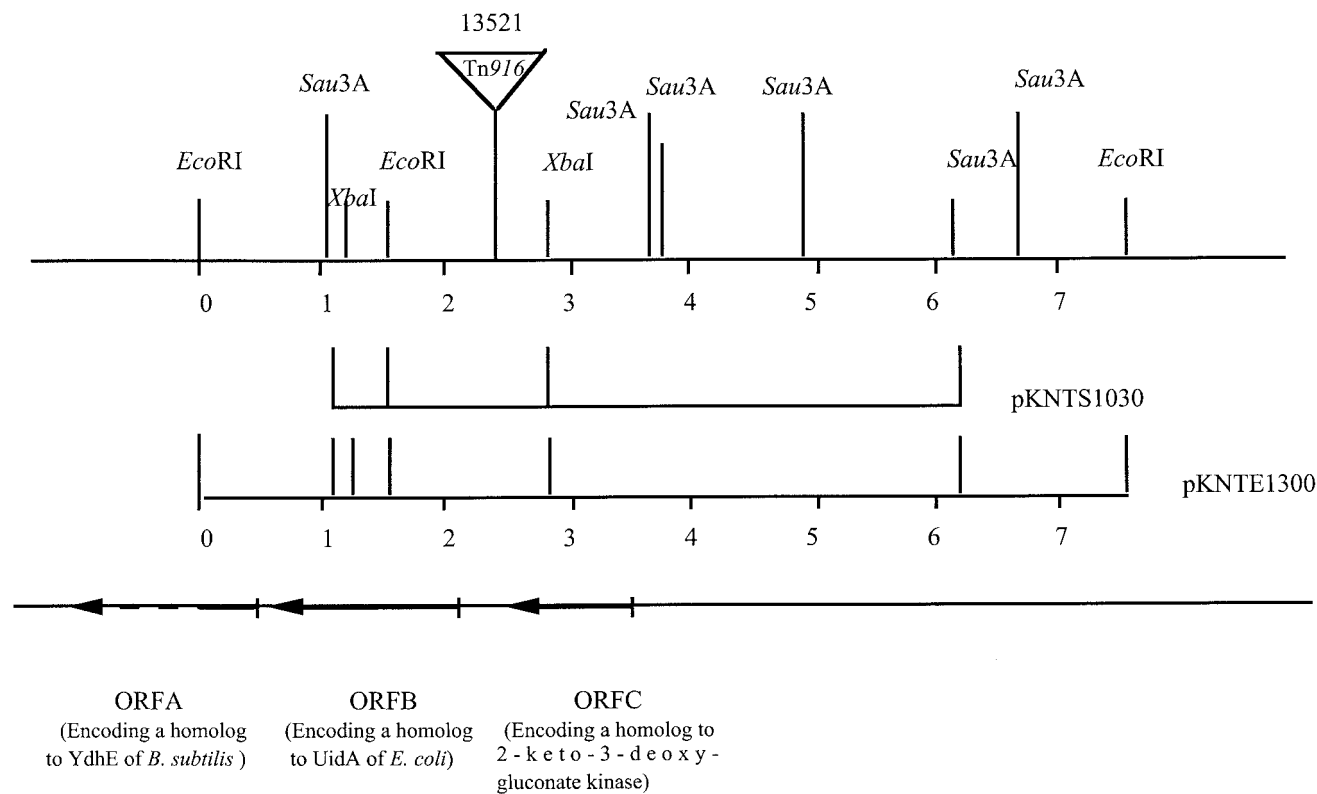


FIG. 4. Partial restriction map of pKNT1300 and pKNTS1030 showing the location of Tn916 in mutant 13521. The solid arrows indicate the locations of the various ORFs identified by sequence analysis. The scale is in kilobases.

grew more rapidly than the wild-type strain alone (data not shown). These results suggested that the *ydaD* and/or *ydcF* ortholog is implicated in the oxidative stress response of *C. perfringens*.

To determine whether the *ydaD*-like and *ydcF*-like genes alone are implicated in resistance to H₂O₂, each gene was independently disrupted by an antibiotic cassette. The *ydaD*

ortholog was disrupted by simple recombination using the suicide plasmid pKNT2073 (*ermC'*), and the *ydcF* ortholog was inactivated using the recombinant suicide plasmid pUC2071 (*catP*). The sensitivities of exponentially growing and stationary-phase cells of the knockout mutants to H₂O₂ were evaluated in anaerobic conditions (Fig 6). Although no significant differences were observed in the sensitivities of the wild-type

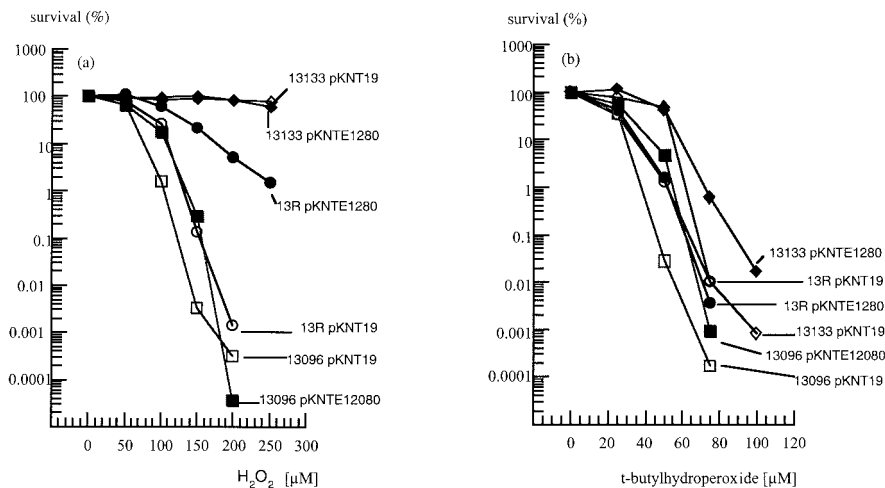


FIG. 5. Sensitivities of 13R, 13096, and 13133 containing plasmid pKNT1280 (solid symbols) or pKNT19 (open symbols) to H₂O₂ and t-BH. The results are means based on three experiments. Symbols: ○, strain 13R/pKNT19; ●, strain 13R/pKNT1280; □, strain 13096/pKNT19; ■, strain 13096/pKNT1280; ◇, strain 13133/pKNT19; ◆, strain 13133/pKNT1280.

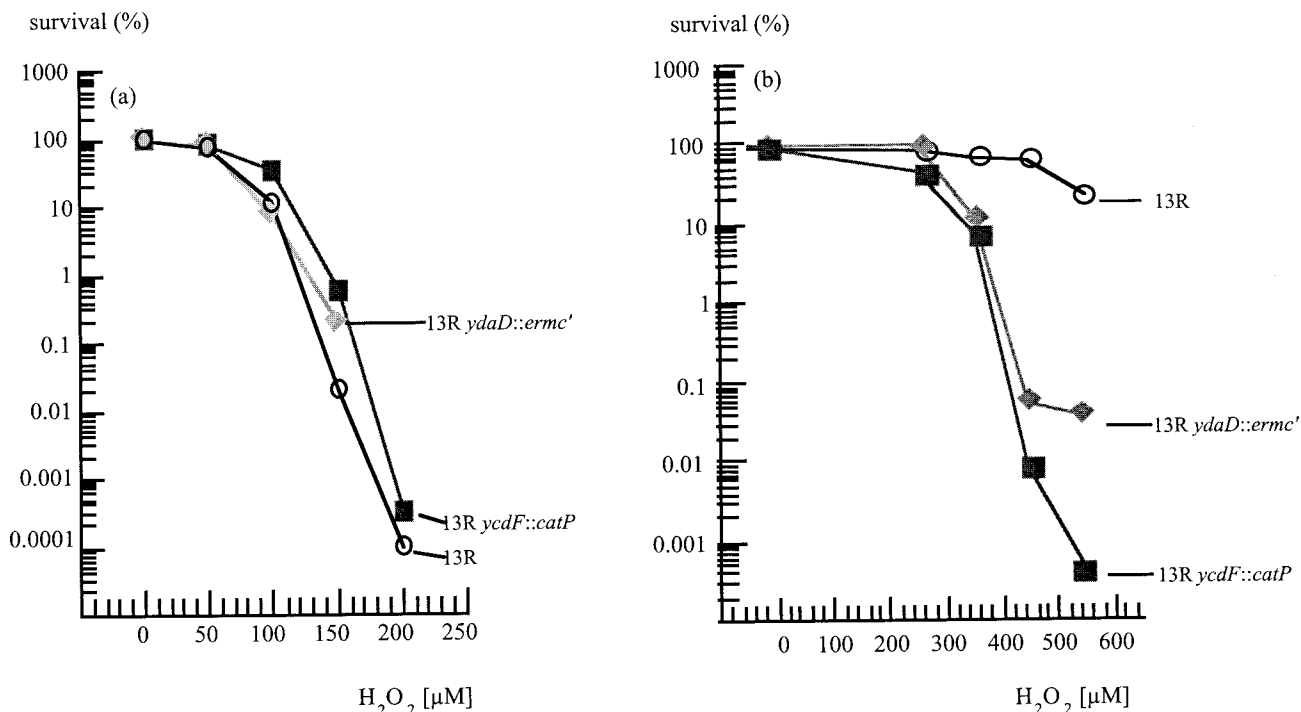


FIG. 6. Survival curves for *C. perfringens* strain 13R and for the knockout mutants 13R *ycdF* ortholog::*catP* and 13R *ydaD* ortholog::*ermc'*, treated with H₂O₂ in anaerobic conditions. The data are means based on two experiments. Symbols: ○, strain 13R; ■, strain 13R *ycdF* ortholog::*catP*; ◆, strain 13R *ydaD* ortholog::*ermc'*. (a) Exponentially growing cells; (b) stationary-phase cells.

strain and the two knockout mutants in the exponential phase of growth (Fig. 6a), the *ycdF::catP* and *ydaD::ermc'* strains were both much more sensitive to H₂O₂ in the stationary phase (Fig. 6b). These results are in good agreement with those obtained in solid medium (Table 2). In anaerobic conditions, only the stationary-phase mutant cells were more sensitive to HO[•]- and ROO[•]-generating compounds than the wild-type strain (diameters of inhibition for H₂O₂, 1.62 to 1.65 cm, compared with 1.27 cm for the wild type; diameter of inhibition for t-TB, 2.05 cm, compared with 1.61 cm for the wild type). However, when the knockout mutants were exposed to air for 30 min, the diameters of inhibition were larger for H₂O₂, t-TB, and PL both for exponential-phase cells and for stationary-phase cells.

Similar complementation experiments were performed with resistant strain 13133, which also contained a copy of Tn916 that was within the *ydaD* ortholog but was integrated in the

orientation opposite that in 13096. The presence of pKNT1280 or pKNT19 did not affect the survival curves for strain 13133 (Fig. 5). As YdaD and YcdF have both been individually implicated in the oxidative stress response, we hypothesized that the resistant phenotype of this *ydaD* mutant can be explained by a polar effect on expression of the *ycdF* gene. Transcription of *ycdF* may be driven from a hypothetical outward promoter within the Tn916 transposon or from a promoter created by insertion of the transposon into the chromosome. Nevertheless, we cannot rule out the possibility that another dominant mutation was selected during the screening procedure.

Role of the *uidA* ortholog of *C. perfringens* in the oxidative stress response. In strain 13521, Tn916 was located upstream of a gene encoding a putative β-glucuronidase, suggesting that the corresponding gene may not be expressed in the mutant. To evaluate the importance of the role of this gene in the oxidative stress response, a null mutant was constructed by

TABLE 2. Inhibition diameters in various oxidative stress conditions for *C. perfringens* strain 13R, for 13R *ydaD* ortholog::*ermc'*, and for 13R *ycdF* ortholog::*catP* in solid MCP medium

Inhibitor	Conditions	Inhibition diam (cm)					
		Stationary phase			Exponential phase		
		Strain 13R	Strain 13R <i>ycdF::catP</i>	Strain 13R <i>ydaD::ermc'</i>	Strain 13R	Strain 13R <i>ycdF::catP</i>	Strain 13R <i>ydaD::ermc'</i>
H ₂ O ₂	Anaerobic	1.27 ^a	1.62	1.65	1.65	1.70	1.57
t-BT	Anaerobic	1.61	2.05	2.05	1.85	2.05	2.00
H ₂ O ₂	Aerobic	1.66	2.10	2.10	1.75	2.40	3.00
t-BT	Aerobic	1.70	1.95	2.00	1.68	2.17	1.95
PL	Aerobic	1.50	2.05	1.95	2.08	2.85	2.90

^a Mean based on four measurements of growth inhibition diameters on the same plate.

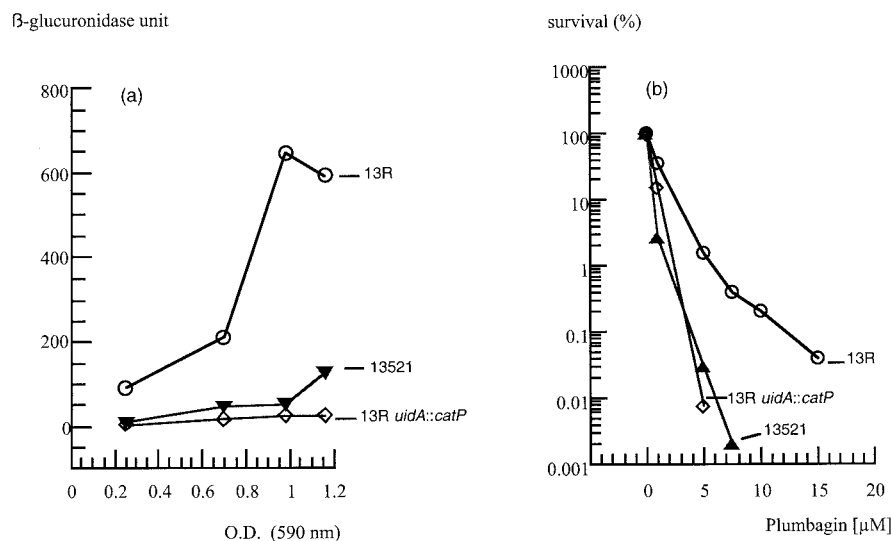


FIG. 7. (a) β -Glucuronidase activities of 13R, 13521, and 13R *uidA::ermc'*. (b) Sensitivities of 13R, 13521, and 13R *uidA::ermc'* to various concentrations of PL. Symbols: \circ , strain 13R; \blacktriangle , strain 13521; \diamond , strain 13R *uidA::catP*.

inserting a *catP* cassette into the *uidA* gene (*uidA::catP*). At all stages of growth in TGY medium, the two mutated strains possessed less than 10% of the β -glucuronidase activity of the wild-type strain cultivated in the same conditions (Fig. 7a). Both 13521 and the mutant containing the *catP*-disrupted gene were hypersensitive to PL (Fig. 7b).

Role of the prismane protein in the oxidative stress response. To confirm that the prismane protein was implicated in the oxidative stress response, the prismane-encoding gene was disrupted by an *ermc'* cassette (Prismane::*ermc'*). The sensitivities to H_2O_2 and PL of the mutant strain were compared to

those of the same strain harboring the wild-type prismane gene cloned on pJIR418 (Fig. 8). In anaerobic conditions, the knockout mutant was more resistant to H_2O_2 than the complemented strain. In contrast, both strains were very sensitive to PL, suggesting that the overproduction of the protein was toxic to the cells in the presence of oxygen.

DISCUSSION

The conjugative properties of the 18-kb Tn916 transposon, which carries a tetracycline resistance gene (*tetM*), were first

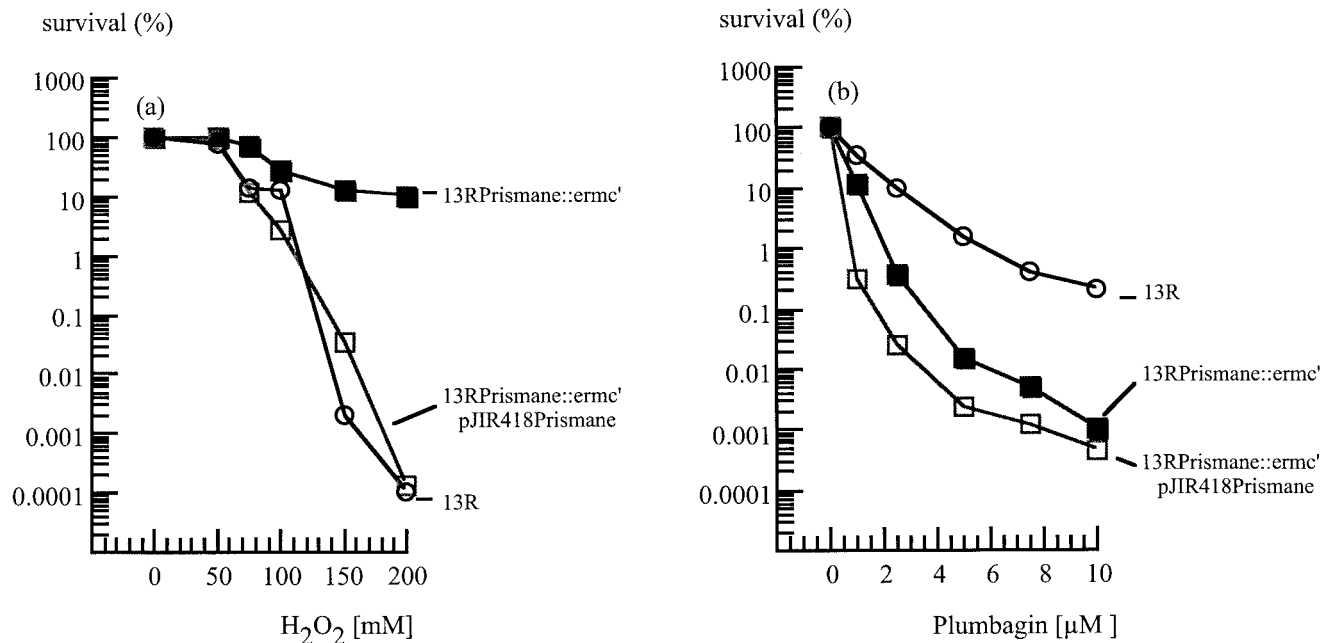


FIG. 8. Survival of strain 13R Prismane::*ermc'* and the same strain complemented with a wild-type prismane gene cloned onto pJIR418, treated with H_2O_2 in anaerobic conditions (a) and with PL (b). Symbols: \circ , strain 13R; \blacksquare , strain 13R Prismane::*ermc'*; \square , strain 13R Prismane::*ermc'* complemented with a wild-type prismane gene cloned onto pJIR418.

used to inactivate *C. perfringens* genes by Kaufmann et al. (14). Insertional mutants have also been obtained by introducing the pBR322-derived plasmid pAM120, which contains the entire Tn916 transposon, into *C. perfringens* by electroporation (18). This useful tool for inactivating *C. perfringens* genes has some limitations, and the following problems have been encountered in other studies. (i) Although Tn916 can insert itself into numerous chromosomal sites, insertion is not entirely random and tends to occur in A+T-rich regions (usually in intergenic regions for low-G+C-content genomes) (31). (ii) Tn916 can also insert itself at multiple sites within the same strain, which complicates analysis of the mutants obtained by this method. For example, only 12 (28%) of the 42 mutants selected in this study contained a single copy of Tn916, a percentage in the range (25 to 37%) observed by Kaufmann et al. (14). (iii) Tn916 possesses the ability to invert within a particular insertion site during growth of *Enterococcus faecium* DPC3675 in broth (23). This property could theoretically hinder PCR-based sequence disruption techniques.

Despite these difficulties, we obtained Tn916 insertional mutants of *C. perfringens* strain 13R with lower resistance and higher resistance to oxidative stress. In four mutants (13133, 13050, 13096, and 13548) the transposon was located in the same gene, which probably formed an operon with a second gene. The first gene of the operon probably encodes a short-chain alcohol dehydrogenase, and the second gene probably encodes a glucose-1-dehydrogenase. The equivalent genes in *B. subtilis*, *ydaD* and *ycdF*, were recently shown to belong to the stationary-phase stress regulon controlled by alternative sigma factor B, which is activated by heat, ethanol, and salt stress or by energy starvation (25). The two encoded proteins are proposed to be involved in generation of NADPH, which is required to maintain the intracellular redox balance in stress conditions. In *E. coli*, the principal intracellular reducing agent, NADPH, is produced mainly by the pentose phosphate pathway by glucose-6-phosphate dehydrogenase, which is under control of the SoxRS regulatory system and is activated in response to elevated concentrations of O_2^{0-} (for a review, see reference 35). Thus, in *C. perfringens* the *ydaD* and *ycdF* homologs might play a role similar to that of glucose-6-phosphate dehydrogenase in *E. coli*. In *C. perfringens*, reduction of oxidized thiol groups of proteins is likely to occur via reduced glutathione. This compound is indeed present at low, but significant levels in this microorganism (21), and a gene exhibiting a high degree of similarity with the gamma-glutamylcysteine synthetase gene of *E. coli*, whose product is implicated in the synthesis of glutathione, has been detected in strain 13R (data not shown). Thus, it is believed that the high concentrations of NADPH required to recycle oxidized glutathione to its reduced form are provided by activation of the *ydaD-ycdF* operon.

In the resistant mutant 13055, a gene encoding an ATP-dependent RNA helicase, which possesses a typical DEAD-box consisting of the Asp-Glu-Ala-Asp amino acid motif (16), was disrupted by insertion of the Tn916 transposon element. According to Luking et al. (16), RNA helicases are ubiquitous. In eukaryotic cells, RNA helicases play a pivotal role in rRNA processing, the initiation of translation, nuclear mRNA export, and mRNA degradation. They also have been implicated in stabilization of mRNA during cell development and differen-

tiation (16). RNA helicases have also been found in prokaryotes, and some of them are involved in regulation of the response to cold stress. For example, in the cyanobacterium *Anabaena* sp., the DEAD-box CrhC RNA helicase protein is specifically produced in response to cold stress. It is therefore absent from cells grown at 30°C and accumulates rapidly when the temperature drops below 25°C (3, 42). Likewise, the product of the *csdA* gene of *E. coli* plays a critical role in cellular adaptation to low temperatures (for a review see reference 37). This 70-kDa cold shock DEAD box protein is produced only when the temperature is shifted from 37 to 15°C, and it is a major ribosome-associated protein at low temperatures (13). According to Jones et al. (13), the CsdA protein, which possesses a helix-destabilizing activity, increases the translational efficiency of mRNAs by unwinding stable secondary structures formed at low temperatures. It is thus possible that such an RNA helicase protein is involved in the regulation of oxidative stress, although its exact role remains to be defined.

The two other newly discovered proteins implicated in resistance to oxidative stresses are the atypical iron sulfur prismane protein (for a review see reference 12) and β -glucuronidase. The prismane protein was initially discovered in the sulfate-reducing bacterium *D. vulgaris* (34) and has since been shown to be present in the genomes of a wide range of microorganisms, such as *Methanococcus jannaschii*, *E. coli*, *Acidithiobacillus ferrooxidans*, *T. maritima* (strain MSB8), and *Methanobacterium thermoautotrophicum* (strain Delta H). According to Cooper et al. (4), the prismane protein contains two unique types of Fe/S clusters: a typical [4Fe-4S] cubane cluster and an unusual asymmetric hybrid cluster, with both μ_2 -sulfido and μ_2 -oxo bridges, that is ligated to the protein via three cysteine residues, a cysteine persulfide, two monodentate glutamate residues, and a histidine residue. Although the function of the protein remains to be determined, the open structure of this novel cluster could be the site of catalytic activity. Further studies are necessary to determine the role of the prismane protein in the oxidative stress response of *C. perfringens*.

β -Glucuronidase plays an important role in the oxidative stress response in eukaryotic cells (30). This is because uronic acid serves as a detoxifying agent by aiding in the excretion of phenols, steroids, and aromatic carboxylic acids in the form of alkyl or aryl D-glucosiduronic acids, thus avoiding potential cellular damage. Likewise, the antioxidant activity of D-glucuronate, an intermediate in the conversion of D-glucose to L-ascorbic acid (or vitamin C), is of central importance in protection against radicals. Involvement of such an enzyme in the oxidative stress response has never been described in bacteria, and its precise role remains to be clarified.

In conclusion, screening of Tn916-mutated *C. perfringens* strain 13 genes led to identification of a set of genes implicated in the oxidative stress response. Some of these genes (e.g., *ydaD* and *ycdF*) have been identified in other microorganisms, but others (e.g., *uidA*, *ydaB*, and *ydb*) were novel. We have started to evaluate the various proteins produced in the complex response to oxidative stress in an anaerobic organism. It was difficult to allocate the function of some of these proteins, because of the wide variety of potential oxidative stress conditions encountered by anaerobic bacteria, the intracellular conversion of the reactive oxygen species, and the complex regulation of this vital stress response. However, the fact that most

of the genes are also present in a large number of other genomes suggests that our understanding of the oxidative stress response of *C. perfringens* can be extended to other microorganisms.

ACKNOWLEDGMENTS

We thank Stewart Cole for his support and advice throughout this work and his team for helping us with the sequencing experiments.

REFERENCES

- Azeddoug, H., J. Hubert, and G. Reyssset. 1992. Stable inheritance of shuttle vectors based on plasmid pIM13 in a mutant strain of *Clostridium acetobutylicum*. J. Gen. Microbiol. **138**:1371–1378.
- Balch, W. E., G. E. Fox, L. J. Magrum, C. R. Woese, and R. S. Wolfe. 1979. Methanogens: reevaluation of a unique biological group. Microbiol. Rev. **43**:260–296.
- Chamot, D., W. C. Magee, E. Yu, and G. W. Owttrim. 1999. A cold shock-induced cyanobacterial RNA helicase. J. Bacteriol. **181**:1728–1732.
- Cooper, S. J., C. D. Garner, W. R. Hagen, P. F. Lindley, and S. Bailey. 2000. Hybrid-cluster protein (HCP) from *Desulfovibrio vulgaris* (Hildenborough) at 1.6 Å resolution. Biochemistry **39**:15044–15054.
- Dupuy, B., and A. L. Sonenshein. 1998. Regulated transcription of *Clostridium difficile* toxin genes. Mol. Microbiol. **27**:107–120.
- Gawron-Burke, C., and D. B. Clewell. 1982. A transposon in *Streptococcus faecalis* with fertility properties. Nature **300**:281–284.
- Gawron-Burke, C., and D. B. Clewell. 1984. Regeneration of insertionally inactivated streptococcal DNA fragments after excision of transposon Tn916 in *Escherichia coli*: strategy for targeting and cloning of genes from gram-positive bacteria. J. Bacteriol. **159**:214–221.
- Geissmann, T. A., M. Teuber, and L. Meile. 1999. Transcriptional analysis of the rubrerythrin and superoxide dismutase genes of *Clostridium perfringens*. J. Bacteriol. **181**:7136–7139.
- Gille, G., and K. Sigler. 1995. Oxidative stress and living cells. Folia Microbiol. (Prague) **40**:131–152.
- Hanahan, D. 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166**:557–580.
- Heidelberg, J. F., J. A. Eisen, W. C. Nelson, R. A. Clayton, M. L. Gwinn, R. J. Dodson, D. H. Haft, E. K. Hickey, J. D. Peterson, L. Umayam, S. R. Gill, K. E. Nelson, T. D. Read, H. Tettelin, D. Richardson, M. D. Ermolaeva, J. Vamathevan, S. Bass, H. Qin, I. Dragoi, P. Sellers, L. McDonald, T. Utterback, R. D. Fleishmann, W. C. Nierman, and O. White. 2000. DNA sequence of both chromosomes of the cholera pathogen *Vibrio cholerae*. Nature **406**:477–483.
- Johnson, M. K. 1998. Iron-sulfur proteins: new roles for old clusters. Curr. Opin. Chem. Biol. **2**:173–181.
- Jones, P. G., M. Mitta, Y. Kim, W. Jiang, and M. Inouye. 1996. Cold shock induces a major ribosomal-associated protein that unwinds double-stranded RNA in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **93**:76–80.
- Kaufmann, P., Y. Lehmann, and L. Meile. 1996. Conjugative transposition of Tn916 from *Enterococcus faecalis* and *Escherichia coli* into *Clostridium perfringens*. Syst. Appl. Microbiol. **19**:35–39.
- Lehmann, Y., L. Meile, and M. Teuber. 1996. Rubrerythrin from *Clostridium perfringens*: cloning of the gene, purification of the protein, and characterization of its superoxide dismutase function. J. Bacteriol. **178**:7152–7158.
- Luking, A., U. Stahl, and U. Schmidt. 1998. The protein family of RNA helicases. Crit. Rev. Biochem. Mol. Biol. **33**:259–296.
- Lumppio, H. L., N. V. Shenvi, A. O. Summers, G. Voordouw, and D. M. Kurtz. 2001. Rubrerythrin and rubredoxin oxidoreductase in *Desulfovibrio vulgaris*: a novel oxidative stress protection system. J. Bacteriol. **183**:101–108.
- Lyrstis, M., A. E. Bryant, J. Sloan, M. M. Awad, I. T. Nisbet, D. L. Stevens, and J. I. Rood. 1994. Identification and molecular analysis of a locus that regulates extracellular toxin production in *Clostridium perfringens*. Mol. Microbiol. **12**:761–777.
- Mahony, D. E., and T. I. Moore. 1976. Stable L-forms of *Clostridium perfringens* and their growth on glass surface. Can. J. Microbiol. **22**:953–959.
- Monod, M., C. Denoya, and D. Dubnau. 1986. Sequence and properties of pIM13, a macrolide-lincosamide-streptogramin B resistance plasmid from *Bacillus subtilis*. J. Bacteriol. **167**:138–147.
- Newton, G. L., K. Arnold, M. S. Price, C. Sherrill, S. B. Delcardayre, Y. Aharonowitz, G. Cohen, J. Davies, R. C. Fahey, and C. Davis. 1996. Distribution of thiols in microorganisms: mycothiol is a major thiol in most actinomycetes. J. Bacteriol. **178**:1990–1995.
- O'Brien, D. K., and S. B. Melville. 2000. The anaerobic pathogen *Clostridium perfringens* can escape the phagosome of macrophages under aerobic conditions. Cell. Microbiol. **2**:505–519.
- O'Keefe, T., C. Hill, and R. P. Ross. 1999. *In situ* inversion of the conjugative transposon Tn916 in *Enterococcus faecium* DPC3675. FEMS Microbiol. Lett. **173**:265–271.
- Pan, N., and J. A. Imlay. 2001. How does oxygen inhibit central metabolism in the obligate anaerobe *Bacteroides thetaiotaomicron*? Mol. Microbiol. **39**:1562–1571.
- Petersohn, A., H. Antelmann, U. Gerth, and M. Hecker. 1999. Identification and transcriptional analysis of new members of the sigma (B) regulon in *Bacillus subtilis*. Microbiology **145**:869–880.
- Poyart, C., P. Berche, and P. Trieu-Cuot. 1995. Characterization of superoxide dismutase genes from gram-positive bacteria by polymerase chain reaction using degenerate primers. FEMS Microbiol. Lett. **131**:41–45.
- Privitera, G., A. Dublanchet, and M. Sebald. 1979. Transfer of multiple antibiotic resistance between subspecies of *Bacteroides fragilis*. J. Infect. Dis. **139**:97–101.
- Rood, J. I., and S. T. Cole. 1991. Molecular genetics and pathogenesis of *Clostridium perfringens*. Microbiol. Rev. **55**:621–648.
- Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
- Schwartz, N. B. 1997. Carbohydrate metabolism II: special pathways, p. 335–359. In M. T. Delvin (ed.), Textbook of biochemistry with clinical correlations, 4th ed. Wiley-Liss, New York, N.Y.
- Scott, J. R., F. Bringel, D. Marra, G. Van Alstine, and C. K. Rudy. 1994. Conjugative transposition of Tn916: preferred targets and evidence for conjugative transfer of a single strand and for a double-stranded circular intermediate. Mol. Microbiol. **11**:1099–1108.
- Scott, P., and J. I. Rood. 1989. Electroporation-mediated transformation of lysotaphin-treated *Clostridium perfringens*. Gene **82**:327–333.
- Sloan, J., T. A. Warner, P. T. Scott, T. L. Bannam, D. I. Berryman, and J. I. Rood. 1992. Construction of a sequenced *Clostridium perfringens*-*Escherichia coli* shuttle plasmid. Plasmid **27**:207–219.
- Stokkermans, J. P., W. A. van den Berg, W. M. van Dongen, and C. Veeger. 1992. The primary structure of a protein containing a putative [6Fe-6S] prismane cluster from *Desulfovibrio desulfuricans* (ATCC 27774). Biochim. Biophys. Acta **1132**:83–87.
- Storz, G., and J. A. Imlay. 1999. Oxidative stress. Curr. Opin. Microbiol. **2**:188–194.
- Takami, H., K. Nakasone, Y. Takaki, G. Maeno, R. Sasaki, N. Masui, F. Fuji, C. Hiram, Y. Nakamura, N. Ogasawara, S. Kuhara, and K. Horikoshi. 2000. Complete genome sequence of the alkaliphilic bacterium *Bacillus halodurans* and genomic sequence comparison with *Bacillus subtilis*. Nucleic Acids Res. **28**:4317–4331.
- Thieringer, H. A., P. G. Jones, and M. Inouye. 1998. Cold shock and adaptation. Bioessays **20**:49–57.
- Touati, D. 1997. Superoxide dismutase in bacteria and pathogen protists, p. 447–493. In J. G. Scandalios (ed.), Oxidative stress and the molecular biology of antioxidant defenses. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Trinh, S., V. Briolat, and G. Reyssset. 2000. Growth response of *Clostridium perfringens* to oxidative stress. Anaerobe **6**:233–240.
- Xiong, J., D. M. Kurtz, Jr., J. Ai, and J. Sanders-Loehr. 2000. A hemerythrin-like domain in a bacterial chemotaxis protein. Biochemistry **39**:5117–5125.
- Yanisch-Perron, C., J. Vieira, and J. Messing. 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33**:103–119.
- Yu, E., and G. W. Owttrim. 2000. Characterization of the cold stress-induced cyanobacterial DEAD-box protein CrhC as an RNA helicase. Nucleic Acids Res. **28**:3926–3934.